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Cystic Fibrosis Transmembrane Conductance Regulator Controls Lung Proteasomal Degradation and Nuclear Factor-κB Activity in Conditions of Oxidative Stress

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Cystic fibrosis is a lethal inherited disorder caused by mutations in a single gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein, resulting in progressive oxidative lung damage. In this study, we evaluated the role of CFTR in the control of ubiquitin-proteasome activity and nuclear factor (NF)-κB/IκB-α signaling after lung oxidative stress. After a 64-hour exposure to hyperoxia-mediated oxidative stress, CFTR-deficient ($cftr^{-/-}$) mice exhibited significantly elevated lung proteasomal activity compared with wild-type ($cftr^{+/+}$) animals. This was accompanied by reduced lung caspase-3 activity and defective degradation of NF-κB inhibitor IκB-α. *In vitro*, human CFTR-deficient lung cells exposed to oxidative stress exhibited increased proteasomal activity and decreased NF-κB-dependent transcriptional activity compared with CFTR-sufficient lung cells. Inhibition of the CFTR Cl^- channel by $CFTR_{inh\text{-}172}$ in the normal bronchial immortalized cell line 16HBE14o− increased proteasomal degradation after exposure to oxidative stress. Caspase-3 inhibition by Z-DQMD in CFTR-sufficient lung cells mimicked the response profile of increased proteasomal degradation and reduced NF-κB activity observed in CFTR-deficient lung cells exposed to oxidative stress. Taken together, these results suggest that functional CFTR Cl^- channel activity is crucial for regulation of lung proteasomal degradation and NF-κB activity in conditions of oxidative stress. ***(Am J Pathol 2008, 172:1184–1194; DOI: 10.2353/ajpath.2008.070310)***

Cystic fibrosis (CF), a lethal hereditary disease, is caused by mutations in a single gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which has been shown to be a cAMP-activated chloride channel[1,2] and to regulate the activity of other ion channels in lung epithelial cells.[3] The main cause of morbidity and mortality in patients with CF is attributable to progressive lung dysfunction. Oxidative stress has been identified as an early complication in the airways of infants and young children with CF.[4–8] Elevated levels of oxidized lipids and proteins are detected in patients with CF in both serum and airway secretions. They are associated with the severity of the lung disease as assessed by lung function parameters and chest radiography scores.[6,9,10] In addition to this increased oxidative damage, there is also evidence of a constitutive decrease in the glutathione concentration in the epithelial lining fluid of CF patients.[8,11] Further, using two CF lung models, CFTR-deficient mice and a CF patient-derived lung cell line, recent studies suggest that dysfunctional CFTR leads to an increase in mitochondrial oxidative stress characterized by a deficiency in mitochondrial glutathione steady-state levels and increased levels of reactive oxygen species (ROS) in CFTR-deficient lung epithelial cells.[12] We showed previously that elevated ROS production in CFTR-deficient lung cells was associated with a low level of apoptotic cell death and reduced caspase-3 activity as compared to normal lung cells.[13]

It is well established that regulation of the oxidant state within the cell modulates survival signaling pathways. In addition to several reports linking the activation of nuclear factor (NF)-κB to the control of cell growth, apoptosis,

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and cell-cycle progression, recent studies have clearly demonstrated that NF-κB activation is a key step in protecting the lung epithelium from hyperoxia-mediated oxidative damage and in increasing the overall survival of the neonatal rodent model.[14,15] The canonical model for NF-κB function postulates that NF-κB is anchored and inactivated in the cytoplasm by association with the NF-κB inhibitor IκB-α in quiescent lung epithelial cells.[16–18] NF-κB usually exists as a heterodimeric complex of p50 and p65/RelA subunits.

In CF, we and others[13,19–24] have shown exaggerated NF-κB activation in CF human primary airway epithelial cells, lung cell lines, and mouse models that lead to an elevated inflammatory response induced by various proinflammatory stimuli. Inflammatory mediators such as bacteria activate NF-κB in lung epithelial cells through rapid phosphorylation of the IκB-α (p-IκB-α) inhibitor by specific IκB kinases (IKK1/2).[25,26] Then P-IκB-α becomes the target for ubiquitination and proteolytic degradation, respectively, by E3 ubiquitin-ligases and the proteasome machinery.[27,28] Degradation of phospho-IκB-α results in nuclear translocation of NF-κB and activation of its numerous target genes.[26,29] Modulation of the NF-κB transcriptional activity through increased proteasome and caspase activity has been reported in numerous epithelial and nonepithelial cell types.[30–32] In the present study, we examined the effect of oxidative stress on proteasome and caspase-3 activities in lungs of CFTR-deficient mice as well as in a CF patient-derived lung epithelial cell line as compared to CFTR-sufficient model systems. We specifically addressed how these selective changes in activity could in turn influence the NF-κB/IκB-α signaling pathway.

Materials and Methods

Generation of Adult CFTR$^{-/-}$ Mice

Experiments were performed on wild-type (WT) C57BL/6 and CFTR-deficient (cftr$^{-/-}$) C57BL/6 *cftr*^{tm1UNC} male mice (16 to 20 weeks of age) obtained from the Centre de Distribution, Typage, et Archivage Animal, Centre National de la Recherche Scientifique (Orléans, France). The CF homozygous mutant mice were originally obtained by a targeted mutation in the *cftr* gene with insertion of a neomycin-resistance cassette into exon 10 via homologous recombination.[33] WT and cftr$^{-/-}$ mice were bred on corn cob pellet bedding and fed a standard rodent chow. An osmotic laxative containing polyethylene glycol (Transipeg; Roche-Nicholas, Gaillard, France) was provided continuously in the drinking water to prevent intestinal obstruction that occurs in CF mice (Eckman). Mice had free access to drinking water and were maintained in a sterile environment with a 12-hour light-dark cycle. All animal procedures were performed in accordance with institutional veterinary ethical guidelines.

Oxidative Stress Lung Injury

To induce oxidative lung injury, as previously characterized,[34] mice were exposed to 95% O_2 for 64 hours in a sealed Plexiglas chamber, at 22 to 24°C. The O_2 and CO_2 levels in the chamber were monitored with an O_2 and CO_2 analyzer. The oxygen flow rate was adjusted to 1.5 L/minute. The CO_2 level was always kept below 0.5%. After a 64-hour exposure, WT and CF mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg), and after exsanguination, the chest was opened, the lung excised, plunged into liquid nitrogen, and stored at −80°C for subsequent RNA and protein analyses. For histochemistry, the lungs were inflated with 10% formalin at a pressure of 5 cm H_2O, excised, and fixed in 10% formalin at room temperature for a minimum of 4 days. Fixed lungs were then mounted in paraffin and 5 μmol/L sections were prepared.

Immunohistochemistry

Active caspase-3 was analyzed by immunostaining in lungs of CFTR$^{-/-}$ and WT mice. Lung sections were deparaffinized and rehydrated in ethanol. After antigen retrieval with a 10 mmol/L citric acid solution (pH 6), slides were blocked with 100% fetal bovine serum. A polyclonal rabbit antibody against active caspase-3 was obtained from R&D Systems (Lille, France) and incubated with the samples for 1 hour at room temperature at a dilution of 1:250 in 1% milk, 0.1% bovine serum albumin, and 0.005% Tween 20 in phosphate-buffered saline (PBS). Slides were then washed with 1% milk, 0.1% bovine serum albumin, and 0.005% Tween 20 in PBS. Binding of anti-caspase-3 was detected using a biotinylated secondary antibody (Becton Dickinson, Le-Pont-de-Claix, France) at a dilution of 1:300 for 30 minutes at room temperature. The slides were then incubated with the streptavidin-peroxidase complex (DakoCytomation) for 30 minutes at room temperature followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DakoCytomation). Slides were then counterstained with hematoxylin and mounted.

Cell Culture and Oxidative Stress Conditions

The CF patient-derived lung epithelial cell system that we used was IB3-1 cells (CFTR genotype ΔF508/W1282X), and the IB3-1-derived cell line S9 that was stably transduced to achieve low-level expression of full-length WT CFTR. The CFTR-deficient IB3-1 lung cells have been characterized previously[35,36] and the CFTR-sufficient S9 lung cells have been shown to have phenotypic correction of a wide range of CF phenotypes.[37,38] Both cell lines were purchased from the American Type Culture Collection (LGC Promochem SARL, Strasbourg, France) and cultured in minimal essential medium with Earle's salts and L-glutamine, supplemented with 10% decomplemented fetal bovine serum and 100 U/ml of penicillin-streptomycin in a humidified CO_2 incubator (37°C, 5% CO_2). Oxidative stress conditions were achieved by growing cells in 5% CO_2 and 95% O_2 at 37°C in a sealed, humidified chamber for 24 hours, which led to the production of cellular ROS, as previously described.[13] In some experiments, confluent

CFTR-deficient IB3-1 cells were preincubated at 27°C for 24 hours before their exposure to control or oxidative stress conditions at 27°C.

Total Protein Extraction

Total protein lysates from either murine whole lungs or cultured cells were incubated in lysis buffer for 1 hour (250 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 0.1% Nonidet P-40, 50 mmol/L Hepes supplemented with 1 mmol/L dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml phenylmethyl sulfonyl fluoride, 2 mmol/L sodium pyrophosphate, 1 mmol/L sodium orthovanadate), and centrifuged for 10 minutes at 4°C at maximum speed (14,000 rpm). Supernatants containing the total intracellular protein extract were removed and the protein concentration was determined using the Bradford assay (protein assay kit; Bio-Rad, Marnes-la-Coquette, France).

Analysis of the Activity and Inhibition of Caspase-3

Active caspase-3 was evaluated in cultured CFTR-sufficient and CFTR-deficient cells using the Caspase-Glo 3/7 assay according to the manufacturer's instructions (Promega, Lyon, France). Briefly, cells were cultured under either basal or oxidative stress conditions as described above. At the indicated time points, cells were washed twice with PBS and incubated with Caspase-Glo reagent for 1 hour in the dark, and the luminescence was measured with a luminometer according to the manufacturer's instruction (Perkin Elmer, Courtaboeuf, France). Blank values were subtracted and the *n*-fold increase in protease activity was determined by comparing the levels of luciferase activity of control cells with those of cells exposed to oxidative stress. Equal numbers of cells were analyzed by counting a parallel set of cells and determining the total cell number for each sample. Caspase-3 inhibition was obtained by incubating cells either with the large caspase inhibitor Z-VAD-FMK or with the specific caspase-3 inhibitor Z-DQMD-FMK, each used at 5 μmol/L (WR International, Fontenay sous bois, France). Cells were treated with or without caspase inhibitor and cultured under oxidative stress conditions. After 24 hours, the inhibition of the caspase-3 activity was measured using the Caspase-Glo 3/7 assay as described above.

Proteasome-Proteolytic Activity Assay

Proteasomal degradation was measured using the proteasome activity assay kit provided by Chemicon (Euromedex, Mundolsheim, France). Briefly, 15 μg of total protein extracts were added to the commercial assay buffer in the presence of the proteasome substrate, a synthetic fluorogenic substrate Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (LLVY-AMC) in a final reaction volume of 100 μl. Samples were incubated at 37°C for at least 1 hour in obscurity. The cleavage of the labeled substrate by the proteasome machinery results in free AMC, which is quantified by fluorescence using a 380/460-nm filter set of a fluorometer. A standard curve was prepared with known dilutions of AMC. For the experiments on proteasome inhibition, cultured CFTR-sufficient and CFTR-deficient lung cells were treated for 24 hours with 300 nmol/L MG132 (Sigma, St. Quentin Fallavier, France). As previously reported, cell respiration was unaffected by MG132 treatment as determined using the MTT assay (data not shown[13]). In some experiments, CFTR-sufficient 16HBE14o− cells (a gift from Dieter C. Gruenert, California Pacific Medical Center Research Institute, San Francisco, CA) were preincubated with the CFTR inhibitor ($CFTR_{inh\text{-}172}$, Calbiochem) for 0.5 hour or 48 hours before oxidative stress experiments.[24,39–41] During the experiment, $CFTR_{inh\text{-}172}$ cells were maintained in the medium during control and oxidative stress conditions.

Western Blot Analysis

Equal amounts of total protein extracts were loaded on 12% sodium dodecyl sulfate-polyacrylamide gels and electrophoresed. Samples were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with the appropriate antibody. The specific antibody against IκB-α was from Santa Cruz Biotechnology (Le Perray en Yvelines, France) and antibodies against polyubiquitinated proteins, UBCh5 and UBCh9, were from Biomol (Tebu, Le Perray en Yvelines, France). The secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG and the signal was detected by the enhanced chemiluminescence method (Amersham ECL; GE, Saclay, France). Equal loadings were confirmed by blotting with an antibody to β-actin (Sigma).

Enzyme-Linked Immunosorbent Assay (ELISA)

The amount of the phosphorylated form of IκB-α (p-IκBα) was measured in total protein extracts, using a modification of a commercially available ELISA kit, IκB-α ActivELISA (BioCarta, San Diego, CA). Briefly, a flat-bottom, 96-well microtiter plate was coated with capture antibody for 24 hours at 4°C. Wells were washed three times with wash buffer and samples or standard added and incubated at 4°C for 4 hours. After three washes, the antibody for detection was added and incubated for 1 hour at room temperature. The plate was further washed three times, and streptavidin-horseradish peroxidase was added and incubated for an additional 1 hour. The plate was then read at 450 nm in an automated microplate reader (Bio-Tek Instruments, Winooski, VT). The standard curve was generated using half-dilutions of p-IκB-α from 0 to 1000 μg/ml, provided by the manufacturer (BioCarta).

Cell Transfection

Transient transfection experiments were performed in both CFTR-deficient and CFTR-sufficient lung cells, using Exgen 500 reagent (Fermentas, Euromedex, Mudol-

sheim, France) according to the manufacturer's instructions, and harvested 72 hours after transfection, after 24 hours of exposure to either basal or oxidative stress conditions. The relative NF-κB transcriptional activity in response to oxidative stress was assessed using the reporter plasmid NF-κB-Luc (Stratagene, Agilent Technologies, Massy, France). This reporter plasmid contains five NF-κB binding elements 3′(TGGGGACTTTCCGC)5′ upstream of the minimum promoter region that drives expression of the firefly luciferase reporter gene. The NF-κB-Luc plasmid as well as the pRL-TK (Promega) plasmid were transfected into both CFTR-deficient and CFTR-sufficient lung cells using Exgen 500 reagent (Fermentas, Euromedex, France) according to the manufacturer's instructions. The pRL-TK plasmid contains the herpes simplex virus thymidine kinase promoter upstream of the Renilla luciferase gene and was monitored in comparison to the normalize firefly luciferase activity. The NF-κB promoter activity in transfected cells was measured using the dual-luciferase reporter assay system kit (Promega) following the manufacturer's instruction. To monitor NF-κB localization, the YFP-p65NF-κB fusion protein was prepared as described previously[42] and transfected into cells grown on glass slides. After culture under the appropriate conditions, cells were fixed *in situ* in PFA 4% for 25 minutes at 4°C, washed, and rehydrated in 0.1 mol/L PBS at pH 7.4. Subcellular localization of YFP-p65NF-κB was observed using an Axiophovert 200 microscope (Zeiss, Le Pecq, France). Representative fields of each cell line under different conditions were observed using epifluorescence and Nomarski differential interference illumination (magnification, ×630).

Statistical Analysis

All data are expressed as means ± SD of at least three different experiments, and *n* indicates the number of experiments. The statistical difference was determined using the Student's *t*-test or one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Results

Modified Activities of Caspase-3 and Proteasome in Response to Oxidative Stress in CF

We previously showed that ROS production in CFTR-deficient lung cells results in a loss in detectable levels of the cell cycle inhibitor $p21^{Cip1/Waf1}$, which was rescued by treatment with the MG132 proteasome inhibitor.[13] By comparing the effect of oxidative stress in CFTR-deficient lung cells to normal lung cells, we also demonstrated that the rescue of $p21^{Cip1/Waf1}$ counteracted lowered apoptotic cell death together with a slighter increase in the caspase-3 activity.[13] These results prompted us to investigate whether oxidative stress resulted in any CF-specific regulation of caspase-3 and proteasome-proteolytic activity through the use of *in vivo* and *in vitro* complementary models of CFTR-deficient mice compared to WT mice, and a human CFTR-deficient lung cell line compared to the same but corrected CFTR-sufficient lung cell line.

After exposure to oxidative stress, we observed that the change in the activities of both caspase-3 and of proteasome were similar in two CF lung models (Figure 1). Indeed, as revealed by immunohistological staining, we observed that oxidative stress resulted in a strong increase in active caspase-3 in lung of WT mice, with intense staining in the bronchi (Figure 1A), whereas caspase-3 staining remained low in the lungs of CFTR-deficient mice (Figure 1B). We consistently observed a sixfold increase in caspase-3 activity in human CFTR-sufficient lung cells under oxidative stress (Figure 1C), as compared to a threefold increase in human CFTR-deficient lung cells (Figure 1D). In parallel, oxidative stress did not change the proteasome activity in lungs of WT mice as in human CFTR-sufficient lung cells (Figure 1, A and C). In contrast, when compared to the basal condition, oxidative stress led to a marked increase in proteasome activity both in lungs of CFTR-deficient mice and in human CFTR-deficient lung cells (Figure 1, B and D).

Defect in the Regulation of the NF-κB Inhibitor IκB-α in Response to Oxidative Stress in CF

It has been repeatedly shown that accelerated proteasomal degradation could regulate the activation of NF-κB through phosphorylation or ubiquitination of the NF-κB inhibitor IκB-α.[30–32] Exposure of WT mice to oxidative stress resulted in a marked decrease in the level of lung IκB-α (Figure 2A). In contrast, in CFTR-deficient mice, no significant change in the level of lung IκB-α was observed on exposure to oxidative stress, although the basal level of IκB-α is greater than in WT mice (Figure 2B). Consistently, the level of IκB-α remained unchanged in human CFTR-deficient lung cells (Figure 2D) whereas a marked decrease in IκB-α was observed in human CFTR-sufficient lung cells on oxidative stress (Figure 2C). Taken together, these data reveal that in response to oxidative stress, degradation of NF-κB inhibitor IκB-α is defective in two CFTR-deficient lung models.

Suppression of NF-κB-Dependent Transcriptional Activity in Response to Oxidative Stress in CF

We next explored whether the lack of IκB-α degradation in CFTR-deficient lung models was specifically associated with a change in the overall regulation of the NF-κB/IκB-α signaling. We first chose to analyze the level of phosphorylated IκB-α (p-IκB-α) that is known as the target form for ubiquitination and proteolytic degradation by the proteasome-proteolytic machinery.[27,28] As shown in Figure 3A, oxidative stress resulted in a significant increase in the level of P-IκB-α protein as observed in

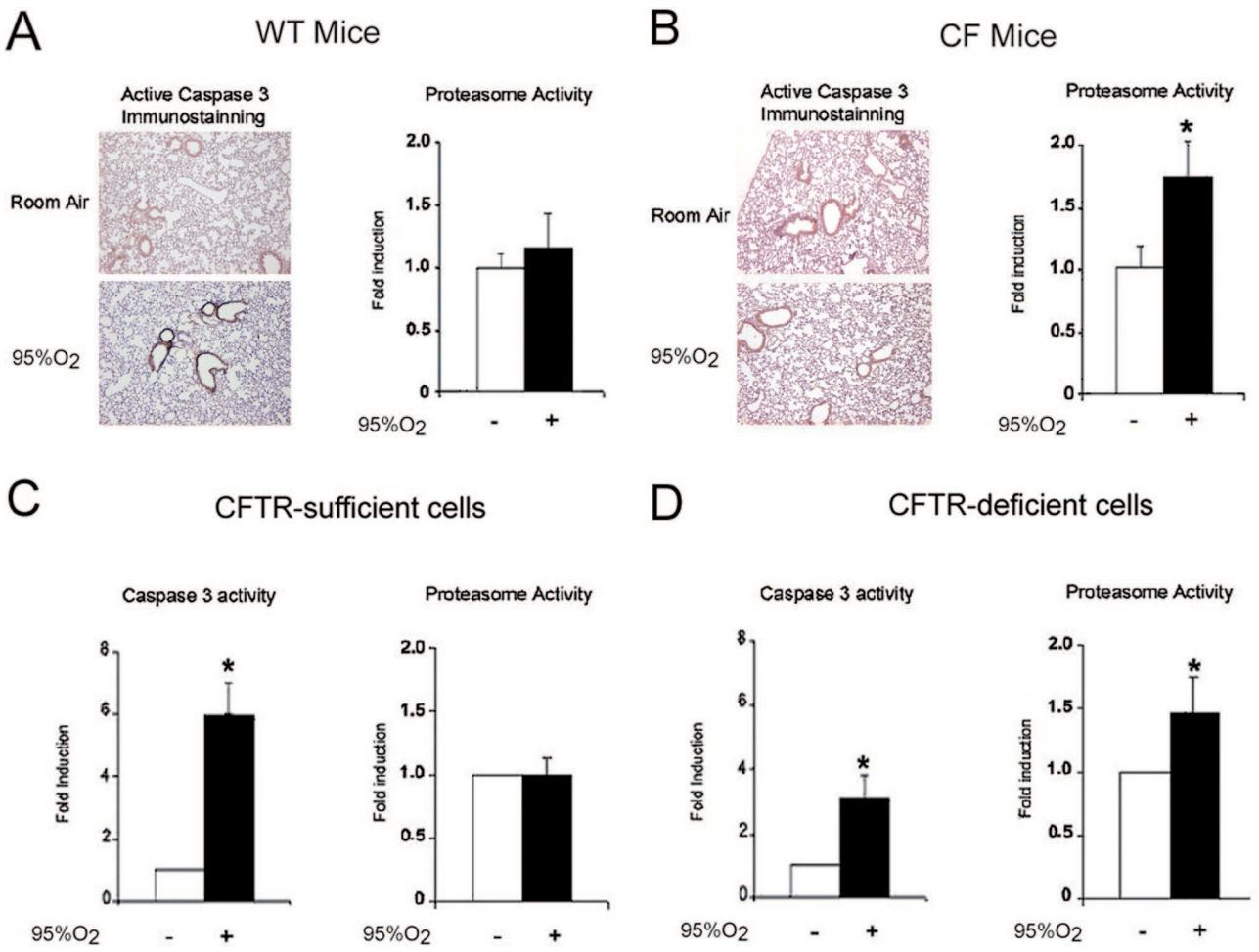

Figure 1. Activities of caspase-3 and proteasome are modified in response to oxidative stress in CFTR-sufficient versus CFTR-deficient *in vivo* and *in vitro* systems. **A** and **B:** The effect of oxidative stress on the caspase-3 and proteasome activities in lungs of WT and cftr$^{-/-}$ (CF) mice. **Left:** Images of immunohistochemical staining of active caspase-3 were representative of immunostainings for two WT and two CF mice placed either under basal conditions or in 95% O_2. **Right:** The proteasome activity assay was determined from the specific cleavage of the LLVY-AMC substrate by the proteasome. The enzymatic activity is plotted as the fold induction relative to the basal condition. Results are expressed as the mean ± SD of four animals [eight WT and eight CF mice split into two equal groups exposed to either room air ($n = 4$) or to 95% O_2 ($n = 4$)]. **C** and **D:** The effect of oxidative stress on the caspase-3 and proteasome activities in CFTR-sufficient versus CFTR-deficient lung epithelial cells. Subconfluent cells were placed either under basal conditions or in 95% O_2 for 24 hours. **Left:** The caspase-3 enzyme activity in cell lines was assayed by the cleavage of a luminescent substrate by active caspase-3 in total lysates and is presented as a fold induction compared to the basal condition. Values are means ± SD of four independent experiments in either cell line. **Right:** The proteasome activity was assayed as in **A** and **B** in at least four independent experiments in either cell type. *$P < 0.05$ compared to the basal condition.

CFTR-sufficient lung cells, whereas no change was detected in CFTR-deficient lung cells (Figure 3B). Thus, our data suggest that the regulation of IκB-α in response to oxidative stress in CF takes place not only at the proteolytic level (Figure 2), but also at the phosphorylation level (Figure 3).

Because the amount of p-IκB-α can condition NF-κB nuclear translocation via direct interaction with the p65NF-κB subunit, we therefore investigated whether the lack of an increase in P-IκB-α could translate into differential regulation of p65 nuclear translocation in CFTR-deficient lung cells. Transfection of cells with a p65-YFP reporter plasmid enabled monitoring of the subcellular localization of p65. Although oxidative stress induced NF-κB nuclear translocation in CFTR-sufficient lung cells, as assessed by the presence of a p65-YFP fusion protein within the nucleus (Figure 3C), no significant nuclear localization of the fusion protein was detected in CFTR-deficient lung cells (Figure 3D).

Then, we explored whether changes in both cell lines the amount of p-IκB-α and nuclear localization of p65-NF-κB subunit were associated with modified NF-κB-dependent transcriptional activity. To this end, both CFTR-deficient and CFTR-sufficient lung cells were transiently transfected with a NF-κB-transcription reporter gene in basal and oxidative stress conditions (Figure 3, E and F). As shown in Figure 3E, exposure to oxidative stress in CFTR-sufficient lung cells resulted in a significant increase of NF-κB-dependent transcriptional activity whereas in CFTR-deficient cells, the NF-κB-dependent transcription was ~3.5-fold reduced under oxidative stress when compared with basal condition (Figure 3F). Thus, the suppression of NF-κB-dependent transcriptional activity observed in oxidative-stressed CFTR-deficient cells matched with the lack of increased level of p-IκB-α (Figure 3B). Interestingly, when we partially restored CFTR function in cell membrane by incubating CF cells at 27°C[43,44] before exposure to oxidative stress, we

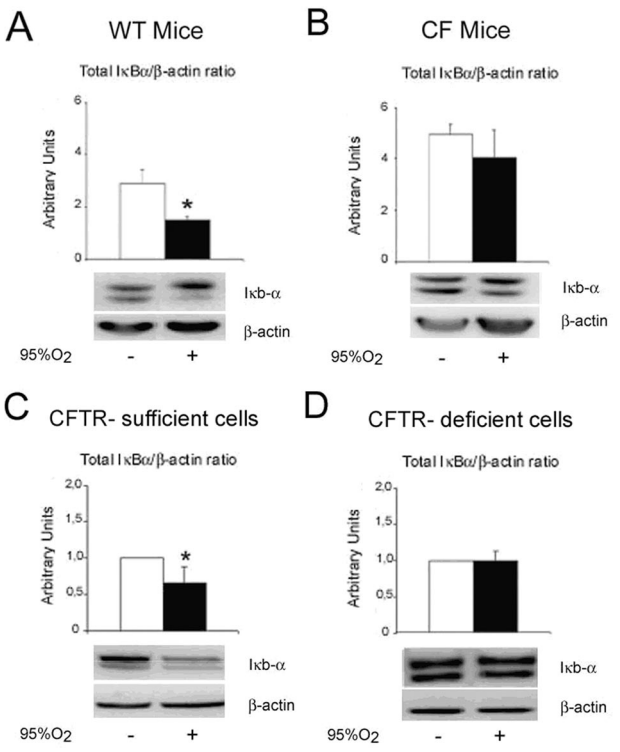

Figure 2. Level of IκB-α protein is decreased *in vivo* as *in vitro* under oxidative stress in CFTR-sufficient systems but not in CFTR-deficient systems. **A–D:** The effect of oxidative stress on the level of IκB-α protein was analyzed in lungs from WT and CF mice (**A** and **B**, respectively) or in CFTR-sufficient and CFTR-deficient cells (**C** and **D**, respectively). A typical blot of IκB-α is shown for each system. As a control for equal loading, the same blot was stripped and reprobed with an antibody to β-actin. Densitometric analysis of four independent experiments is shown as a graph of fold induction of the level of IκB-α protein expression in the basal condition and in 95% O_2. $^*P <$ 0.05 compared to basal condition.

did not observe any significant changes in NF-κB-dependent transcriptional activity, indicating a potential role of the functional CFTR in the modulation of NF-κB activity in oxidative-stressed CF cells.

Increased Proteasome Activity in Oxidative-Stressed CF Cells Is Attributable to CFTR Deficiency

To further analyze the effect of oxidative stress on the proteasome machinery, we choose to document the modulation of ubiquitilated protein and ubiquitin-conjugating enzymes UBCh9 and UBCh5 after treatment with the potent proteasome inhibitor MG132 in either cell type in basal or in oxidative condition. After testing the efficiency of MG132 treatment on the proteasome activity (data not shown), we demonstrated that neither MG132 treatment nor oxidative condition affect the ubiquitination process, or the level of both the UBCh9 and UBCh5 ubiquitin-conjugating enzyme (Figure 4, A and B).

To further demonstrate a role of CFTR Cl^- channel function in the increased proteasome activity in response to oxidative stress, we treated the normal bronchial immortalized epithelial cell line 16HBE14o− with the potent CFTR inhibitor, $CFTR_{inh\text{-}172}$[24,39–41] for two 0.5-hour and 48-hour periods before oxidative stress. As shown in Figure 4, C and D, we demonstrated that the inhibition of the CFTR Cl^- function in 16HBE14o− cells caused a significant increase of proteasomal degradation in oxidative stress condition.

Finally, we also demonstrated that a 27°C pretreatment of CFTR-deficient cells leads to the abolition of the increased proteasome activity observed in the oxidative stress condition. As shown in Figure 4E, in both CFTR-corrected S9 cells, normal 16HBE14o− cells and 27°C pretreated IB3-1 cells, we observed no significant variation of the proteasome activity in response to oxidative stress.

Restoration of NF-κB-Dependent Transcriptional Activity in CFTR-Deficient Lung Cells under Oxidative Stress by MG132 Treatment

To determine whether the previously observed modifications in the activity of the ubiquitin-proteasome system were involved in the regulation of NF-κB/IκB-α signaling in CFTR-deficient lung cells, we examined the effect of MG132 on both the level of IκB-α and NF-κB-dependent transcriptional activity in CFTR-deficient and CFTR-sufficient lung cells (Figure 5). In basal and oxidative stress conditions, treatment of the CFTR-sufficient lung cells with MG132 resulted in no significant change in the level of IκB-α (Figure 5A) as in the level of NF-κB-dependent transcriptional activity (Figure 5C). In contrast, in CFTR-deficient lung cells, both in basal and oxidative stress condition, MG132 treatment resulted in a marked decrease of level of IκB-α (Figure 5B), and a significant increase in NF-κB-dependent transcriptional activity (Figure 5D). In fact, this latter increase was more pronounced under oxidative stress conditions (fourfold to fivefold increase versus the basal condition), to be finally restored to a similar value to that in CFTR-sufficient lung cells under oxidative stress conditions.

Inhibition of Caspase-3 Activity in CFTR-Sufficient Lung Cells Results in Both Modified Activities of Proteasome and NF-κB-Dependent Transcription Similarly to CFTR-Deficient Lung Cells under Oxidative Stress

It is well established that caspases negatively regulate the proteasome-proteolytic activity[45,46] and activate the NF-κB/IκB-α pathway.[47] We thus hypothesized that the CF signature of an elevated proteasome activity, which was observed, *in vivo*, as *in vitro* on exposure to oxidative stress, might be related to the lower increase in caspase-3 activity that we observed under the same conditions (Figure 1). To test this hypothesis, we choose to use the CFTR-sufficient lung cells in which, in response to oxidative stress, proteasomal activity remained unaffected and NF-κB-dependent

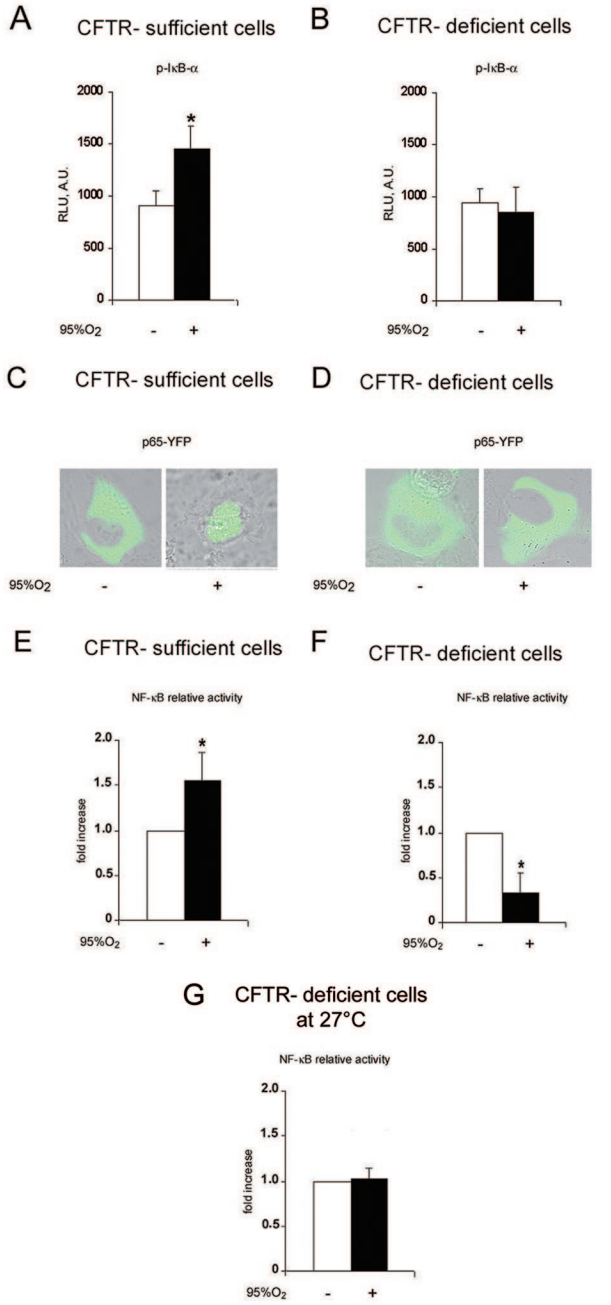

Figure 3. The NF-κB-dependent transcriptional activity is increased in CFTR-sufficient cells but decreased in CFTR-deficient cells under oxidative stress. The activation of NF-κB/IκB-α pathway was studied by analyzing the level of p-IκB-α, the nuclear translocation of p65-NF-κB fusion protein, and the NF-κB-dependent transcriptional activity. **A** and **B:** Level of IκB-α phosphorylation. CFTR-sufficient (**A**) and CFTR-deficient (**B**) cells were cultured under basal or oxidative stress conditions and the level of IκB-α phosphorylation was analyzed by the ELISA method. Results are expressed as the mean relative luminescence units (RLUs) ± SD of at least four experiments in two cultured cell types ($^*P < 0.05$) compared to the control condition. **C** and **D:** Localization of the YFP-p65NF-κB fusion protein. CFTR-sufficient and CFTR-deficient cells were transiently transfected with a plasmid expressing the YFP-p65NF-κB fusion protein and exposed or not to 24 hours of 95% O_2. Images are representative of four independent experiments. **E–G:** NF-κB-dependent transcriptional activity. CFTR-sufficient and CFTR-deficient cells were transiently transfected with a NF-κB-firefly-luciferase reporter construct and a control *R. reniformis*-luciferase vector and exposed or not to 24 hours of 95% O_2. In **G**, transfected CFTR-deficient cells were preincubated at 27°C for 24 hours before their exposure to control or oxidative stress conditions at 27°C. The luciferase activity indicative of NF-κB-dependent transcriptional activity is expressed as a value relative to that observed under basal conditions. Results are shown as the mean ± SD of at least four independent experiments. $^*P < 0.05$ compared to the control condition.

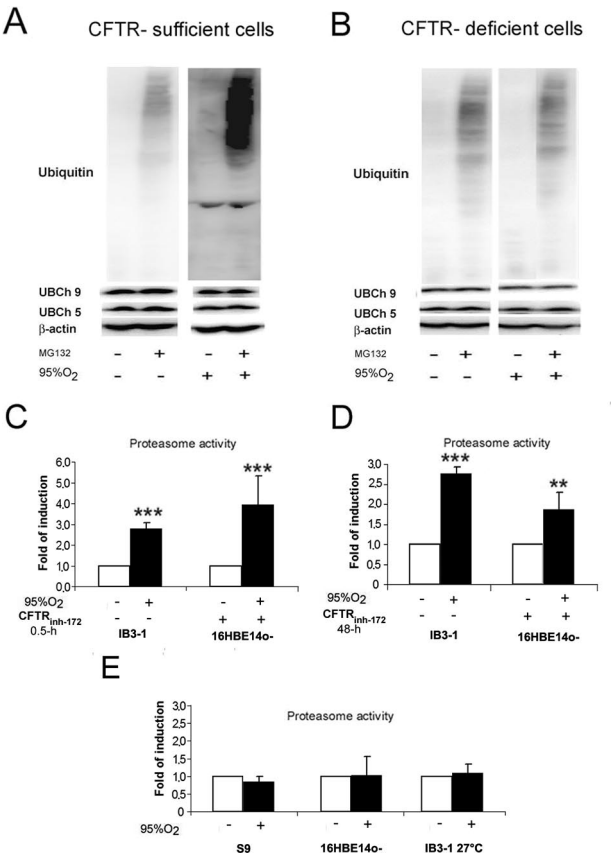

Figure 4. Implication of CFTR deficiency in the modulation of the proteasome activity in response to oxidative stress. **A** and **B:** Western blot analysis of the effect of oxidative stress alone or in combination with a 24-hour treatment of 500 nmol/L MG132 on the rate of ubiquitinated proteins and levels of expression of the ubiquitin-conjugating enzymes UbcH5 and UbcH9. To normalize the quantity of proteins, the same blots were stripped and reprobed with an anti-β-actin antibody. At least four experiments were conducted and a typical blot is shown in the figure. **C** and **D:** Inhibition of CFTR on normal bronchial epithelial cells 16HBE14o− and its consequence on the proteasome activity. 16HBE14o− cells were treated with the specific CFTR inhibitor ($CFTR_{inh\text{-}172}$) at 10 μmol/L for 0.5 hour or 48 hours before exposition to oxidative stress. Proteasome activity is plotted as fold induction relative to the basal condition. Results are expressed as the mean ± SD of at least four independent experiments. $^*P < 0.05$ compared to the control condition. **E:** Effect of a rescue of CFTR on the proteasome activity. CFTR-deficient cells were preincubated at 27°C for 24 hours before exposure to control or oxidative stress conditions at 27°C. Proteasome activity of S9 cells, 16HBE14o−, and IB3-1 cells at 27°C is plotted as fold induction relative to the basal condition. Results are expressed as the mean ± SEM of at least four independent experiments. $^*P < 0.05$ compared to the control condition.

transcription was increased (Figure 3E). We treated CFTR-sufficient lung cells with two distinct caspase-3 inhibitors. Notably, condition of oxidative stress together with Z-VAD-FMK or Z-DQMD-FMK treatment, was sufficient to significantly reduce the NF-κB-dependent transcriptional activity (Figure 6A) and decrease the proteasome-proteolytic activity (Figure 6B) in CFTR-sufficient lung cells to similar levels to those obtained in CFTR-deficient lung cells. Thus, inhibition of caspase-3 activity in CFTR-sufficient lung cells mimic the molecular changes that were reproducibly and significantly observed in CFTR-deficient lung cells when exposed to oxidative stress.

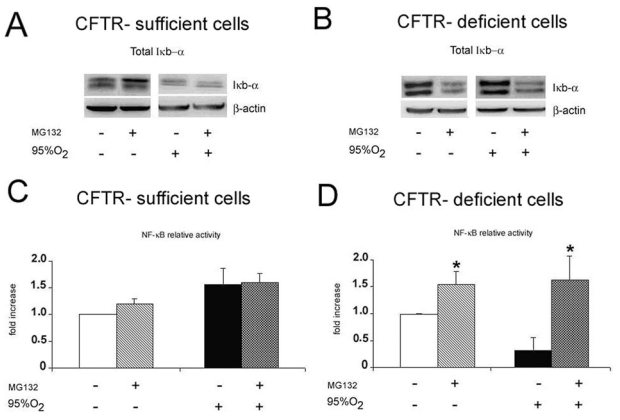

Figure 5. Treatment with MG132 restores regulation of the IκB-α inhibitor and NF-κB-dependent transcription activity in CFTR-deficient cells to a similar level to that in CFTR-sufficient cells**. A** and **B:** Typical blots of the level of the IκB-α protein in cells incubated with 500 nmol/L MG132 are shown. As a control for equal loading, the same blots were stripped and reprobed with an antibody to β-actin. **C** and **D:** The luciferase activity indicative of NF-κB-dependent transcriptional activity in which MG132 is expressed as a value relative to that observed in the basal condition in CFTR-sufficient (**C**), and CFTR-deficient (**D**) cells. Results are shown as the mean ± SD of at least four independent experiments. $^{*}P < 0.05$ compared to the control condition.

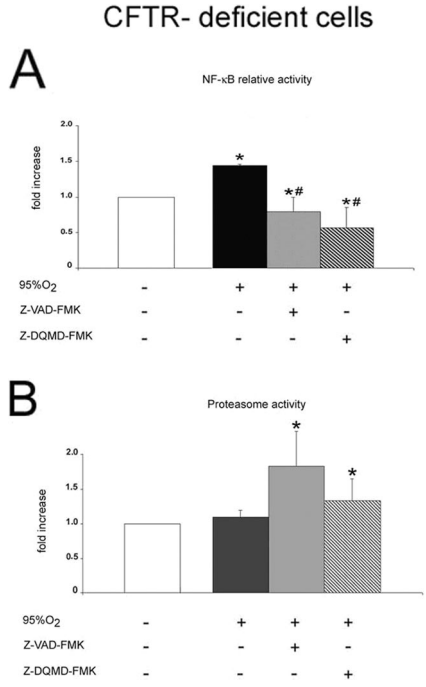

Figure 6. Inhibition of caspase-3 in CFTR-sufficient cells results in the same type of modulation of NF-κB-dependent transcription and proteasome activity as in oxidative-stressed CF systems**. A** and **B:** CFTR-sufficient cells were treated with 5 μmol/L of either the broad range caspase inhibitor Z-VAD FMK or the specific caspase-3 inhibitor Z-DQMD-FMK incubated either in basal or oxidative stress conditions (95% O_2) for a 24-hour period. **A:** Effect of caspase-3 inhibition on NF-κB-dependent transcriptional activity. The luciferase activity indicative of the NF-κB-dependent transcriptional activity of CFTR-sufficient cells under oxidative stress is shown as a value relative to that observed under the basal condition. Results are expressed as the mean ± SD of at least four independent experiments. $^{*}P < 0.05$ compared to room air and $^{*\#}P < 0.05$ compared to 95% O_2. **B:** Effect of caspase-3 inhibition on the proteasome-proteolytic activity. The proteasome activity is given as a value relative to that observed in the basal condition. Results are expressed as the mean ± SD of at least four independent experiments. $^{*}P < 0.05$ compared to the control condition.

Discussion

Control of oxidative stress in the lung of patients is a key objective in the treatment of CF disease. High levels of oxidants in fluids lining the lung epithelium of young and adult patients with CF disease contribute to irreversible lung damage and ultimately to death. New data have reported that oxidative stress suppresses CFTR expression[7] and a CFTR-deficient state in lung epithelium is associated with high levels of cellular ROS production.[12] We recently demonstrated that an increase in ROS production in human CFTR-deficient lung cells promoted higher proteasomal degradation of the cell cycle inhibitor $p21^{CIP1/WAF1}$, lowered caspase-3 activity, and had less apoptosis as compared to normal lung epithelial cell line.[13]

Despite extensive reports on the effects of oxidative stress on the NF-κB activity in the normal lung epithelium,[14,15,48–50] our study is the first to document the effects of oxidative stress on the ubiquitin-proteasome system, caspase-3 activity, and NF-κB/IκB-α signaling in *in vivo* and *in vitro* CF lung models. Our results reveal that oxidative stress in CFTR-deficient models, either murine lungs or a human lung epithelial cell line, led to: 1) increased proteasomal activity, 2) reduced caspase-3 activation, and 3) a suppression of NF-κB-dependent transcriptional activity. Recently, we have reported that in contrast to two WT-CFTR-corrected-S9 and normal 16HBE14o− cell lines, no increase of NF-κB-dependent transcriptional activity attributable to an absence of reduction of IκB-α inhibitor in cytosol was observed in two CF lung epithelial cell lines, IB3-1 and CFBE41o−, after exposure to oxidative stress.[51] In the present study, we also demonstrate that CFTR Cl^- channel inhibition by $CFTR_{inh\text{-}172}$ in normal 16HBE14o− cells exposed to oxidative stress causes an increase of proteasomal activity.

The findings in the present study reveal that ubiquitin-proteasome-dependent proteolysis appeared to be enhanced in both CFTR-deficient models under oxidative stress and was efficiently blocked *in vitro* after treatment with the proteasome inhibitor MG132. Ubiquitination is mediated by an enzymatic cascade, which includes ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes.[52,53] Interestingly, a composite atlas of high-abundance proteins of the CF lung epithelial proteome,[54] using the same CFTR-sufficient cell line as the one used herein, reported up-regulation of enzymes for ubiquitination when compared to CFTR-sufficient cells.[38] Therefore, an excessive accumulation of ubiquitin-protein conjugates[55] and of misfolded ΔF508CFTR together with chaperone proteins such as Hsc70[54,56,57] could conceivably create proteasome dysfunction, in particular, under conditions of oxidative stress. Recent studies have suggested that there is a relationship between the endoplasmic reticulum stress, the unfolded protein response activation, and wtCFTR but not mutant ΔF508CFTR expression regulation in room air conditions.[58] An increase in proteasome activity and a decrease in the NF-κB activity would indicate an increased in endoplasmic reticulum stress in CFTR-deficient lung

cells. However, based on the results obtained between CFTR-sufficient and CFTR-deficient lung cells under conditions of oxidative stress, it is most likely that the increase in proteasomal proteolytic activity seen in CFTR-deficient lung cells results from a loss of CFTR Cl^- channel activity rather that the endoplasmic reticulum stress. However, we cannot rule out a potential effect of induced endoplasmic reticulum stress in our condition of oxidative stress and require further investigation.

In the present study, we also show that the IκB-α phosphorylation and degradation is followed by an increase in the NF-κB activity after treatment of CFTR-deficient lung cells with the proteasome inhibitor MG132 under oxidative stress. Additional regulatory mechanisms can also lead to the activation of NF-κB. One possibility is that the effect of the proteasome inhibitor MG132 may be mediated through modulating signaling pathways such as c-Jun terminal kinases, p38 mitogen-activated protein kinases, and p42/44 mitogen-activated protein kinases[59–61] that are all known to mediate IκB-α degradation and NF-κB activation.[62,63] Another possibility is that proteasome inhibition induces NF-κB activation by a mechanism involving the FasL-Fas pathway in oxidative-stressed CFTR-deficient lung cells. Indeed, proteasome inhibitors have been demonstrated to stimulate this pathway and FasL-Fas ligand interaction can activate NF-κB through the activity of IKK kinases.[64] It is also conceivable that another proteolytic system can mediate the degradation of IκB-α after treatment with the proteasome inhibitor MG132 in oxidative-stressed CFTR-deficient lung cells. One such proteolytic system might be the protease calpains that have been recently described to represent an alternative mechanism for IκB-α degradation and NF-κB activation independently of the ubiquitin-proteasome pathway.[65,66] In addition to calpains, the caspases may play a role in NF-κB-dependent transcriptional activity in CF lung cells because caspase-3 activation can result in IκB-α degradation.[67] Inhibition of proteasome has been shown to induce caspase-3 activation[68] and dual caspase-8 and caspase-9 activation, followed by independent activation of the Fas/caspase-8 pathway.[69] Whether all these effects are caused by inhibition of proteasome activity and how the defective CFTR protein in the lung epithelium in condition of oxidative stress may influence the absence of IκB-α degradation and NF-κB-dependent transcriptional activity awaits further investigation.

As we reported in a previous study,[13] we repeatedly observed a lower increase in caspase-3 activity in both lungs of CFTR-deficient mice and human CFTR-deficient lung cells under oxidative stress. We also demonstrated that inhibition of caspase-3 activity in oxidative stressed, CFTR-sufficient cells could mimic the response profile of increased proteasomal degradation and suppressed NF-κB activity, which appeared as the signature of oxidative stressed CFTR-deficient lung cells. Our data are in good agreement with other reports showing that active caspase-3 negatively regulates proteasome activity in a variety of cell types.[45,46] Together with the direct involvement of NF-κB activation in protecting the lung epithelium from oxidative stress-induced damage,[14,15] our findings shed new light on the understanding of CF lung pathophysiology. Indeed, our results support the hypothesis that in conditions of oxidative stress, a lowered activity of caspase-3, by a mechanism yet to be discovered, is responsible for the increased proteasomal activity and the lack of increased NF-κB-dependent transcriptional activity in CF lung.

In summary, our work reveals a crucial role of functional CFTR Cl^- channel activity in regulating lung proteasomal degradation, caspase-3 activity, and NF-κB-dependent transcriptional activity under oxidative stress conditions. Further studies are now required to determine which of the components of the ubiquitin-proteasome and caspase complexes might be optimal targets to promote an increase in NF-κB-dependent transcriptional activity in the CF lung epithelium but with low toxicity. Such a strategy might provide more tailor-made therapies for patients with CF who are subject to notable deleterious effects of oxidants.

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References

1. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, et al: Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA [published erratum appears in Science 1989 Sep 29;245(4925): 1437]. Science 1989, 245:1066–1073
2. Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC: Identification of the cystic fibrosis gene: genetic analysis. Science 1989, 245:1073–1080
3. Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, Boucher RC: CFTR as a cAMP-dependent regulator of sodium channels. Science 1995, 269:847–850
4. Brown RK, McBurney A, Lunec J, Kelly FJ: Oxidative damage to DNA in patients with cystic fibrosis. Free Radic Biol Med 1995, 18:801–806
5. Witko-Sarsat V, Allen RC, Paulais M, Nguyen AT, Bessou G, Lenoir G, Descamps-Latscha B: Disturbed myeloperoxidase-dependent activity of neutrophils in cystic fibrosis homozygotes and heterozygotes, and its correction by amiloride. J Immunol 1996, 157:2728–2735
6. Kettle AJ, Chan T, Osberg I, Senthilmohan R, Chapman AL, Mocatta TJ, Wagener JS: Myeloperoxidase and protein oxidation in the airways of young children with cystic fibrosis. Am J Respir Crit Care Med 2004, 170:1317–1323
7. Cantin AM, Bilodeau G, Ouellet C, Liao J, Hanrahan JW: Oxidant stress suppresses CFTR expression. Am J Physiol 2006, 290: C262–C270
8. Cantin AM, White TB, Cross CE, Forman HJ, Sokol RJ, Borowitz D: Antioxidants in cystic fibrosis. Conclusions from the CF antioxidant workshop, Bethesda, Maryland, November 11–12, 2003. Free Radic Biol Med 2007, 42:15–31
9. Lagrange-Puget M, Durieu I, Ecochard R, Abbas-Chorfa F, Drai J, Steghens JP, Pacheco Y, Vital-Durand D, Bellon G: Longitudinal study of oxidative status in 312 cystic fibrosis patients in stable state and during bronchial exacerbation. Pediatr Pulmonol 2004, 38:43–49
10. McGrath LT, Mallon P, Dowey L, Silke B, McClean E, McDonnell M, Devine A, Copeland S, Elborn S: Oxidative stress during acute respiratory exacerbations in cystic fibrosis. Thorax 1999, 54:518–523
11. Hull J, Vervaart P, Grimwood K, Phelan P: Pulmonary oxidative stress response in young children with cystic fibrosis. Thorax 1997, 52:557–560
12. Velsor LW, Kariya C, Kachadourian R, Day BJ: Mitochondrial oxidative stress in the lungs of cystic fibrosis transmembrane conductance

regulator protein mutant mice. Am J Respir Cell Mol Biol 2006, 35:579–586
13. Boncoeur E, Tabary O, Bonvin E, Muselet C, Fritah A, Lefait E, Redeuilh G, Clement A, Jacquot J, Henrion-Caude A: Oxidative stress response results in increased p21WAF1/CIP1 degradation in cystic fibrosis lung epithelial cells. Free Radic Biol Med 2006, 40:75–86
14. Franek WR, Morrow DM, Zhu H, Vancurova I, Miskolci V, Darley-Usmar K, Simms HH, Mantell LL: NF-kappaB protects lung epithelium against hyperoxia-induced nonapoptotic cell death-oncosis. Free Radic Biol Med 2004, 37:1670–1679
15. Yang G, Abate A, George AG, Weng YH, Dennery PA: Maturational differences in lung NF-kappaB activation and their role in tolerance to hyperoxia. J Clin Invest 2004, 114:669–678
16. Barnes PJ, Adcock IM: Transcription factors and asthma. Eur Respir J 1998, 12:221–234
17. Blackwell TS, Christman JW: The role of nuclear factor-kappa B in cytokine gene regulation. Am J Respir Cell Mol Biol 1997, 17:3–9
18. Sen R, Baltimore D: Inducibility of kappa immunoglobulin enhancer-binding protein NF-kappa B by a posttranslational mechanism. Cell 1986, 47:921–928
19. Joseph T, Look D, Ferkol T: NF-kappaB activation and sustained IL-8 gene expression in primary cultures of cystic fibrosis airway epithelial cells stimulated with Pseudomonas aeruginosa. Am J Physiol 2005, 288:L471–L479
20. Tabary O, Escotte S, Couetil JP, Hubert D, Dusser D, Puchelle E, Jacquot J: Genistein inhibits constitutive and inducible NFkappaB activation and decreases IL-8 production by human cystic fibrosis bronchial gland cells. Am J Pathol 1999, 155:473–481
21. Venkatakrishnan A, Stecenko AA, King G, Blackwell TR, Brigham KL, Christman JW, Blackwell TS: Exaggerated activation of nuclear factor-kappaB and altered IkappaB-beta processing in cystic fibrosis bronchial epithelial cells. Am J Respir Cell Mol Biol 2000, 23:396–403
22. DiMango E, Ratner AJ, Bryan R, Tabibi S, Prince A: Activation of NF-kappaB by adherent Pseudomonas aeruginosa in normal and cystic fibrosis respiratory epithelial cells. J Clin Invest 1998, 101:2598–2605
23. Saadane A, Soltys J, Berger M: Role of IL-10 deficiency in excessive nuclear factor-kappaB activation and lung inflammation in cystic fibrosis transmembrane conductance regulator knockout mice. J Allergy Clin Immunol 2005, 115:405–411
24. Perez A, Issler AC, Cotton CU, Kelley TJ, Verkman AS, Davis PB: CFTR inhibition mimics the cystic fibrosis inflammatory profile. Am J Physiol 2007, 292:L383–L395
25. Karin M, Ben-Neriah Y: Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 2000, 18:621–663
26. Li Q, Verma IM: NF-kappaB regulation in the immune system. Nat Rev Immunol 2002, 2:725–734
27. Alvarez-Castelao B, Castano JG: Mechanism of direct degradation of IkappaBalpha by 20S proteasome. FEBS Lett 2005, 579:4797–4802
28. May MJ, Ghosh S: Signal transduction through NF-kappa B. Immunol Today 1998, 19:80–88
29. Chen G, Goeddel DV: TNF-R1 signaling: a beautiful pathway. Science 2002, 296:1634–1635
30. Amit S, Ben-Neriah Y: NF-kappaB activation in cancer: a challenge for ubiquitination- and proteasome-based therapeutic approach. Semin Cancer Biol 2003, 13:15–28
31. Ghosh S, Karin M: Missing pieces in the NF-kappaB puzzle. Cell 2002, 109:S81–S96
32. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T: The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell 1994, 78:773–785
33. Snouwaert JN, Brigman KK, Latour AM, Malouf NN, Boucher RC, Smithies O, Koller BH: An animal model for cystic fibrosis made by gene targeting. Science 1992, 257:1083–1088
34. Ahamed K, Epaud R, Holzenberger M, Bonora M, Flejou JF, Puard J, Clement A, Henrion-Caude A: Deficiency in type 1 insulin-like growth factor receptor in mice protects against oxygen-induced lung injury. Respir Res 2005, 6:31–42
35. Zeitlin PL, Lu L, Rhim J, Cutting G, Stetten G, Kieffer KA, Craig R, Guggino WB: A cystic fibrosis bronchial epithelial cell line: immortalization by adeno-12-SV40 infection. Am J Respir Cell Mol Biol 1991, 4:313–319
36. Eidelman O, Srivastava M, Zhang J, Leighton X, Murtie J, Jozwik C, Jacobson K, Weinstein DL, Metcalf EL, Pollard HB: Control of the proinflammatory state in cystic fibrosis lung epithelial cells by genes from the TNF-alphaR/NFkappaB pathway. Mol Med 2001, 7:523–534
37. Srivastava M, Eidelman O, Zhang J, Paweletz C, Caohuy H, Yang Q, Jacobson KA, Heldman E, Huang W, Jozwik C, Pollard BS, Pollard HB: Digitoxin mimics gene therapy with CFTR and suppresses hypersecretion of IL-8 from cystic fibrosis lung epithelial cells. Proc Natl Acad Sci USA 2004, 101:7693–7698
38. Virella-Lowell I, Herlihy JD, Liu B, Lopez C, Cruz P, Muller C, Baker HV, Flotte TR: Effects of CFTR, interleukin-10, and Pseudomonas aeruginosa on gene expression profiles in a CF bronchial epithelial cell Line. Mol Ther 2004, 10:562–573
39. Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C, Galietta LJ, Verkman AS: Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. J Clin Invest 2002, 110:1651–1658
40. Antigny F, Norez C, Becq F, Vandebrouck C: Calcium homeostasis is abnormal in cystic fibrosis airway epithelial cells but is normalized after rescue of F508del-CFTR. Cell Calcium 2008, 43:175–183
41. Muanprasat C, Kaewmokul S, Chatsudthipong V: Identification of new small molecule inhibitors of cystic fibrosis transmembrane conductance regulator protein: in vitro and in vivo studies. Biol Pharm Bull 2007, 30:502–507
42. Tabary O, Boncoeur E, de Martin R, Pepperkok R, Clement A, Schultz C, Jacquot J: Calcium-dependent regulation of NF-(kappa)B activation in cystic fibrosis airway epithelial cells. Cell Signal 2006, 18:652–660
43. Denning GM, Anderson MP, Amara JF, Marshall J, Smith AE, Welsh MJ: Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. Nature 1992, 358:761–764
44. Powell K, Zeitlin PL: Therapeutic approaches to repair defects in deltaF508 CFTR folding and cellular targeting. Adv Drug Deliv Rev 2002, 54:1395–1408
45. Henderson CJ, Aleo E, Fontanini A, Maestro R, Paroni G, Brancolini C: Caspase activation and apoptosis in response to proteasome inhibitors. Cell Death Differ 2005, 12:1240–1254
46. Sun XM, Butterworth M, MacFarlane M, Dubiel W, Ciechanover A, Cohen GM: Caspase activation inhibits proteasome function during apoptosis. Mol Cell 2004, 14:81–93
47. Chaudhary PM, Eby MT, Jasmin A, Kumar A, Liu L, Hood L: Activation of the NF-kappaB pathway by caspase 8 and its homologs. Oncogene 2000, 19:4451–4460
48. Franek WR, Chowdary YC, Lin X, Hu M, Miller EJ, Kazzaz JA, Razzano P, Romashko III J, Davis JM, Narula P, Horowitz S, Scott W, Mantell LL: Suppression of nuclear factor-kappa B activity by nitric oxide and hyperoxia in oxygen-resistant cells. J Biol Chem 2002, 277:42694–42700
49. Rahman I, Biswas SK, Jimenez LA, Torres M, Forman HJ: Glutathione, stress responses, and redox signaling in lung inflammation. Antioxid Redox Signal 2005, 7:42–59
50. Barazzone-Argiroffo C, Pagano A, Juge C, Metrailler I, Rochat A, Vesin C, Donati Y: Glucocorticoids aggravate hyperoxia-induced lung injury through decreased nuclear factor-kappa B activity. Am J Physiol 2003, 284:L197–L204
51. Boncoeur E, Saint Criq V, Roque T, Henrion-Caude A, Gruenert DC, Clement A, Jacquot J, Tabary O: Oxidative stress induces extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase in cystic fibrosis lung epithelial cells: potential mechanism for excessive IL-8 expression. Int J Biochem Cell Biol 2008, 40:432–446
52. Ciechanover A, Iwai K: The ubiquitin system: from basic mechanisms to the patient bed. IUBMB Life 2004, 56:193–201
53. Finley D, Ciechanover A, Varshavsky A: Ubiquitin as a central cellular regulator. Cell 2004, 116:S29–S32
54. Pollard HB, Ji XD, Jozwik C, Jacobowitz DM: High abundance protein profiling of cystic fibrosis lung epithelial cells. Proteomics 2005, 5:2210–2226
55. Younger JM, Ren HY, Chen L, Fan CY, Fields A, Patterson C, Cyr DM: A foldable CFTR{Delta}F508 biogenic intermediate accumulates upon inhibition of the Hsc70-CHIP E3 ubiquitin ligase. J Cell Biol 2004, 167:1075–1085
56. Farinha CM, Amaral MD: Most F508del-CFTR is targeted to degradation at an early folding checkpoint and independently of calnexin. Mol Cell Biol 2005, 25:5242–5252

57. Cyr DM: Arrest of CFTRDeltaF508 folding. Nat Struct Mol Biol 2005, 12:2–3
58. Rab A, Bartoszewski R, Jurkuvenaite A, Wakefield J, Collawn JF, Bebok Z: Endoplasmic reticulum stress and the unfolded protein response regulate genomic cystic fibrosis transmembrane conductance regulator expression. Am J Physiol 2007, 292:C756–C766
59. Madrid LV, Mayo MW, Reuther JY, Baldwin AS Jr: Akt stimulates the transactivation potential of the RelA/p65 subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. J Biol Chem 2001, 276: 18934–18940
60. Hashimoto S, Matsumoto K, Gon Y, Maruoka S, Kujime K, Hayashi S, Takeshita I, Horie T: p38 MAP kinase regulates TNF alpha-, IL-1 alpha- and PAF-induced RANTES and GM-CSF production by human bronchial epithelial cells. Clin Exp Allergy 2000, 30:48–55
61. Orlowski RZ, Small GW, Shi YY: Evidence that inhibition of p44/42 mitogen-activated protein kinase signaling is a factor in proteasome inhibitor-mediated apoptosis. J Biol Chem 2002, 277:27864–27871
62. Romashko J III, Horowitz S, Franek WR, Palaia T, Miller EJ, Lin A, Birrer MJ, Scott W, Mantell LL: MAPK pathways mediate hyperoxia-induced oncotic cell death in lung epithelial cells. Free Radic Biol Med 2003, 35:978–993
63. Lee PJ, Choi AM: Pathways of cell signaling in hyperoxia. Free Radic Biol Med 2003, 35:341–350
64. Russo MP, Bennett BL, Manning AM, Brenner DA, Jobin C: Differential requirement for NF-kappaB-inducing kinase in the induction of NF-kappaB by IL-1beta, TNF-alpha, and Fas. Am J Physiol 2002, 283:C347–C357
65. Han Y, Weinman S, Boldogh I, Walker RK, Brasier AR: Tumor necrosis factor-alpha-inducible IkappaBalpha proteolysis mediated by cytosolic m-calpain. A mechanism parallel to the ubiquitin-proteasome pathway for nuclear factor-kappab activation. J Biol Chem 1999, 274:787–794
66. Shen J, Channavajhala P, Seldin DC, Sonenshein GE: Phosphorylation by the protein kinase CK2 promotes calpain-mediated degradation of IkappaBalpha. J Immunol 2001, 167:4919–4925
67. Reuther JY, Baldwin AS Jr: Apoptosis promotes a caspase-induced amino-terminal truncation of IkappaBalpha that functions as a stable inhibitor of NF-kappaB. J Biol Chem 1999, 274:20664–20670
68. Zhang XM, Lin H, Chen C, Chen BD: Inhibition of ubiquitin-proteasome pathway activates a caspase-3-like protease and induces Bcl-2 cleavage in human M-07e leukaemic cells. Biochem J 1999, 340:127–133
69. Mitsiades N, Mitsiades CS, Poulaki V, Chauhan D, Fanourakis G, Gu X, Bailey C, Joseph M, Libermann TA, Treon SP, Munshi NC, Richardson PG, Hideshima T, Anderson KC: Molecular sequelae of proteasome inhibition in human multiple myeloma cells. Proc Natl Acad Sci USA 2002, 99:14374–14379